Use of real-time reverse transcriptase polymerase chain reaction assay and cell culture methods for detection of swine influenza A viruses

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Objective—To evaluate sensitivity and specificity of a real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay performed on pooled nasal swab specimens, compared with virus isolation performed on individual nasal swab specimens by use of 2 cell culture lines for detection of swine influenza A viruses.

Sample Population—900 nasal swab specimens obtained from pigs at an abattoir and 62 nasal swab specimens submitted for diagnostic testing.

Procedures—Primers were chosen to amplify a conserved portion of the influenza virus matrix gene. Assay sensitivity was initially determined by testing serial dilutions of various subtypes of swine influenza viruses. Sensitivity and specificity were confirmed by use of nasal swab specimens with or without addition of known concentrations of influenza virus and further validated by testing nasal swab specimens obtained through an abattoir surveillance program or submitted for diagnostic testing. Aliquots of specimens were pooled in sets of 10, and results of real-time RT-PCR assays were compared with results of virus isolation of individual specimens in Madin Darby canine kidney (MDCK) and mink lung (Mv1Lu) cells.

Results—Real-time RT-PCR assay was highly specific (100%) and sensitive (88% to 100%). Among the 16 viruses isolated, 3 grew only in Mv1Lu cells and 3 grew only in MDCK cells.

Conclusions and Clinical Relevance—Results indicate that real-time RT-PCR assay is a fast and accurate test for screening numerous nasal swab specimens for swine influenza virus. Some viruses were isolated in only MDCK or Mv1Lu cells, indicating that use of >1 cell line may be required to isolate a broad range of influenza A viruses. (Am J Vet Res 2005;66:119–124)

Infection with influenza A virus is one of the leading causes of respiratory disease in pigs throughout the world[1] and has a substantial economic impact on the swine industry. Influenza virus infections in pigs also present important public health concerns.[2,14] Wild waterfowl and seabirds provide a vast global reservoir of influenza A viruses of all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes.[9,12,21] Viruses from these birds have historically contributed to the generation of novel pandemic human influenza strains.[3] However, because of biochemical differences in the cells’ sialic acid residues, which serve as the virus receptor, the direct transmission of influenza viruses between birds and humans occurs infrequently.[14] In contrast, porcine respiratory cells possess forms of sialic acid[15] that support the entry of both avian and human influenza viruses.[8,16] As such, it has been suggested that pigs serve as intermediary hosts permitting the adaptation of avian influenza viruses to replicate in mammalian cells.[3] Pigs can also serve as mixing vessel hosts in which, because of the segmented nature of the influenza virus genome, influenza viruses can undergo genetic reassortment.[5,7] Reassortment involving the 2 viral surface glycoproteins (HA and NA) can lead to antigenic shifts and the emergence of new viruses. In humans, the pandemics of 1957 and 1968 were caused by reassortant viruses containing mixtures of avian and human virus genes.[7] Among pigs, for nearly 70 years, influenza virus outbreaks in North America were caused almost exclusively by infection with classical swine H1N1 (cH1N1) viruses.[19,20] However, since 1997, reassortant H3N2 (rH3N2), reassortant H1N2 (rH1N2), and reassortant H1N1 (rH1N1) viruses, containing gene segments of human, classical swine, and avian lineages, have emerged and spread widely throughout North America.[20,24] In addition, avian H4N6 (avH4N6) viruses have been isolated from pigs in Canada on a much more limited scale.[3]

Because pigs play such a crucial role in the evolution of influenza viruses and because of the economic importance of influenza virus infections in pigs, it is logical and prudent to perform influenza virus surveillance within swine populations. The methods presently used for diagnosis of influenza virus infections include virus isolation in embryonated chicken eggs and cell culture, antigen detection via fluorescent antibody and ELISA testing, reverse transcriptase polymerase chain reaction (RT-PCR) assay, and serologic analyses.[26-29] The screening of a large number of samples for influenza virus requires an assay that provides accurate, rapid, and sensitive results and that is easy to perform and cost-effective. Real-time RT-PCR has been...
used successfully as a tool to detect infectious agents from a variety of clinical samples. On the basis of the sensitivity of the assay, which makes detection and identification of minuscule amounts of viral nucleic acid possible, a real-time RT-PCR assay meets most of the necessary requirements for influenza virus screening. However, the costs for running real-time PCR assays on a large number of samples, as required for virus surveillance, could be prohibitive, and ultimately it is important to obtain virus isolates for further analyses.

Influenza virus subtypes differ substantially in their HA and NA genes. Polymerase chain reaction assay primers and probes designed for one of these genes would restrict the sensitivity of the assay. In contrast, the sequences of the internal genes, such as the matrix (M), nucleoprotein, and the nonstructural genes, are conserved across influenza A viruses. To establish a real-time RT-PCR assay capable of detecting the wide range of influenza virus strains and subtypes that may infect pigs, we selected primers and a probe from a region of the M gene that is particularly highly conserved across different subtypes and swine, avian, and human evolutionary lineages of influenza A viruses.

We investigated whether real-time RT-PCR assay could be used to initially screen pools of nasal swab specimens before attempting virus isolation from individual nasal swab specimens from pigs. Therefore, the purpose of the study reported here was to evaluate the sensitivity and specificity of a real-time RT-PCR assay performed on pooled nasal swab specimens, compared with virus isolation performed on individual nasal swab specimens in cell culture with 2 cell lines for detection of swine influenza isolate A virus and the most commonly used cell lines for influenza A virus isolation are Madin Darby canine kidney (MDCK) and mink lung (Mv1Lu) cells. To compare the use of MDCK and Mv1Lu cells for swine influenza virus isolation, we inoculated each cell line with the individual nasal swab specimens. Furthermore, because results of other studies indicate that a number of influenza A viruses do not grow in 1 of the 2 cell lines, we used a 1:1 mixture of the 2 cell types to detect a broader variety of influenza viruses than either cell line individually. To assess the use of the mixed MDCK-Mv1Lu cell lines, we infected the 1:1 cell mixture with nasal swab specimens containing influenza viruses previously found to grow in only 1 of the 2 cell lines.

Materials and Methods

Collection and storage of nasal swab specimens—The performance of the assay was evaluated on 90 pools of nasal swab specimens from pigs, each pool consisting of 10 individual specimens. Between November 2001 and April 2002, 900 nasal swab specimens were collected from pigs at a large abattoir in northern Illinois. This facility receives pigs from the 1:1 cell mixture with nasal swab specimens containing influenza viruses previously found to grow in only 1 of the 2 cell lines.

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with 10% fetal bovine serum (FBS), penicillin-streptomycin, and amphotericin B. Before infection, cells were washed twice with MEM and inoculated in duplicate with 100 µL of viral transport medium from a nasal swab specimen. Each plate contained 2 negative (mock-inoculated) and 2 positive (A/Sw/MN/593/99 [H3N2] at 101 TCID50-infected) control wells. Cells were incubated for 1 hour at 37°C; the inoculum was removed; 500 µL of MEM supplemented with 3% bovine serum albumin, 0.01% FBS, penicillin-streptomycin, amphotericin B, and trypsin (TPCK-treated trypsin) (1 µg/µL for MDCK cells, 0.3 µg/µL for Mv1Lu cells) was added; and cells were incubated for 48 hours at 37°C. The presence of virus in each well was determined by immunocytochemical staining by use of a mouse anti-nucleoprotein monoclonal antibody (68D2) as previously described. Virus isolates recovered from only MDCK or Mv1Lu cells were subsequently inoculated on MDCK-Mv1Lu cell mixtures. The MDCK and Mv1Lu cells were brought into suspension by adding 3 mL of 0.25% EDTA-trypsin to the flasks, and the detached cells were counted and mixed at a ratio of 1:1. The cell mixture was grown in 24-well cell culture plates in MEM supplemented with 10% FBS, penicillin-streptomycin, and amphotericin B. Sample inoculation and detection of virus growth were performed in the same manner as for the individual cell lines.

Virus genotyping and limited sequencing—Influenza viruses isolated from either MDCK or Mv1Lu cell cultures were genotyped by conventional RT-PCR assay using H1-, H3-, N1-, and N2-specific primers. To further confirm the identity of the virus isolates, limited nucleotide sequencing of the HA and NA gene PCR amplicons was performed by use of the dye deoxy terminator method.

Statistical analyses—Data between cell cultures testing positive and those testing negative for virus were analyzed by use of a 2-tailed Student t test with unequal variance. Means were considered significantly different at values of P < 0.05. Virus isolation in either MDCK or Mv1Lu cell culture was the gold standard for the presence of influenza virus to which the real-time RT-PCR assay was compared. To determine a threshold cycle (Ct) cutoff value for the real-time RT-PCR assay that would optimally predict which sample pools contained infectious virus, a parametric receiver operating characteristic (ROC) curve analysis was performed by use of the raw Ct values of the pooled abattoir specimens from which viruses were or were not isolated in cell culture. Data were analyzed by use of a commercial statistical computer software program.

Results

Nasal swab specimens—The threshold of the real-time RT-PCR assay (on the basis of the midpoint of the linear region of the amplicon development curve) was defined as normalized reporter signal (Rn) = 0.1. Influenza viruses were isolated in cell culture from 8 of 90 pools of nasal swab specimens collected at the abattoir. Each pool testing positive for virus as determined by real-time RT-PCR assay contained either 1 or 2 individual nasal swab specimens with positive cell culture results. Influenza viruses could not be isolated in 82 of 90 pools. The mean ± SEM Ct value (real-time RT-PCR assay cycle numbers at Rn = 0.1) for pools from which viruses were isolated was 30.6 ± 2.2 (median, 31.0). The mean ± SEM Ct value for pools from which viruses were not isolated was 39.5 ± 1.0 (median, 40.0; Figure 1). The difference in Ct values between pools with positive and negative results for virus isolation was significant (P = 0.005). The real-time RT-PCR assay did not detect influenza virus in 1 pool (Ct = 39) that contained 1 nasal swab specimen determined to be positive by virus isolation.

Calculation of the Ct cutoff value for the real-time RT-PCR assay—An ROC analysis was performed on the Ct values for the pooled abattoir samples from which viruses were or were not subsequently isolated in cell culture. Results of this analysis indicated that the optimal Ct cutoff value to predict which pools would yield viruses in cell culture was ≤ 36.5.

Sensitivity and specificity of the real-time RT-PCR assay for reference viruses—The sensitivity of detection of swine influenza A viruses by use of the M
Gene primers and probe was further determined by serial dilutions of 4 swine influenza viruses of different subtypes in viral transport medium. The minimal levels of detection for the swine influenza virus isolates by use of the previously established optimal Ct cutoff value ($\leq 36.5$) were A/Swine/Wisconsin/235/97 (H1N1), $10^4$ TCID$_{50}$; A/Swine/Minnesota/593/99 (H3N2), $10^3$ TCID$_{50}$; A/Swine/Indiana/9K035/99 (H1N2), $10^6$ TCID$_{50}$; and A/Swine/Ontario/01911-1/99 (avH4N6), $10^3$ TCID$_{50}$. Results of the real-time RT-PCR assay for the nasal swab specimens collected from naïve pigs held in biosafety level 2 isolation were consistently negative.

Nasal swab specimens submitted for diagnostic testing—Influenza viruses were isolated from all 6 pools of nasal swab specimens submitted for diagnostic testing during outbreaks of influenza-like illness among pigs. The mean $\pm$ SEM Ct value for these pools was $24 \pm 1.94$ (median, 24). By use of a Ct cutoff value $\leq 36.5$, the real-time RT-PCR assay was 100% sensitive, compared with virus isolation in cell culture for these specimens. Five of 6 pools contained at least 2 but as many as 9 nasal swab specimens from which virus could be isolated in cell culture. One pool contained only 1 nasal swab specimen that had a positive cell culture result.

Virus isolation and characterization—Sixteen swine influenza viruses were isolated from the abattoir or diagnostic nasal swab specimens. Ten influenza virus isolates grew in MDCK and Mv1Lu cells. These viruses were genotyped as rH3N2, rH1N2, rH1N1, or cH1N1 swine influenza viruses. Three isolates, which were genotyped as rH3N2 or rH1N2, grew only in Mv1Lu cells. Three additional rH1N2 influenza viruses could only be isolated from MDCK cell cultures. Three (2 rH1N2 and 1 rH3N2) of the 6 isolates that grew in only 1 cell line were subsequently inoculated on the MDCK-Mv1Lu cell mixture. All 3 viruses were recovered from the mixed cells. Original nasal swab specimens for the other 3 isolates that grew only in 1 cell line were no longer available for testing on the MDCK-Mv1Lu cell mixture.

Discussion

In the study reported here, we evaluated a real-time RT-PCR assay as a tool to screen pooled nasal swab specimens for influenza virus before attempting virus isolation in cell culture. Our findings indicated that the real-time RT-PCR assay by use of primers and probe directed against a conserved region of the M gene is a sensitive and specific method for the detection of influenza A viruses in nasal swab specimens. In fact, we were able to detect as little as $10^{-1}$ to $10^6$ TCID$_{50}$ of reference swine influenza isolates, including cH1N1, rH3N2, rH1N2, and avH4N6 viruses. This is comparable to the sensitivity reported for detection of influenza A or B viruses by nested RT-PCR assays, using primers specific for the M gene. The ability to detect as little as $10^{-1}$ TCID$_{50}$ of virus may be due to the capability of the real-time RT-PCR assay to detect fewer virions than the minimum number required to initiate a productive infection in cell culture. The real-time RT-PCR assay is also expected to detect noninfectious virus particles, which have been estimated to constitute as much as 90% of some influenza virus preparations.

By use of virus isolation from individual nasal swab specimens in cell culture as the gold standard, the sensitivity of the real-time RT-PCR assay on the pooled nasal swab specimens from healthy pigs collected at the abattoir was 88%. Despite the high sensitivity found for the 4 reference viruses, the real-time RT-PCR assay failed to detect influenza virus in 1 pool that contained 1 nasal swab specimen from which influenza virus was isolated in cell culture. One possible reason for this failure may be the nature of the sample tested. Ribonucleases (RNases) are present in various quantities in specimens collected from the respiratory tract and may gradually digest viral RNA not protected by the viral envelope. Thereby, the sensitivity of the real-time RT-PCR assay may be reduced in clinical specimens that contain large amounts of RNase and a low concentration of viral RNA. Another explanation for the false-negative result could be the presence of PCR assay inhibitors, such as hemoglobin or lactoferrin in the sample. The PCR assay inhibitors were previously reported to be present in about 2% of samples from the respiratory tract, and they can represent a considerable problem in diagnostic PCR-based assays.

Finally, failure to detect the positive specimen with the real-time RT-PCR assay could have also been caused by uneven distribution of virus in the sample. The latter is supported by the fact that the influenza virus could only be isolated from 1 of 4 sample-inoculated cell culture wells. In contrast, the sensitivity of the pooled real-time RT-PCR assay was 100% when the specimens were obtained from pigs with influenza-like illness. The specificity of the assay, determined by lack of amplification of M gene sequence from samples with negative results for virus and confirmation of the identity of the virus isolates as influenza viruses (by subtyping and limited sequencing of the viral genome), was 100% for both sample populations.

An ROC analysis was used to establish an optimal Ct cutoff value to distinguish between pools with positive and negative results because, on rare occasions, negative control samples yielded detectable amplification. This background fluorescence at high cycle numbers was most likely caused by premature probe degradation. Negative controls, consisting of neat transport medium and water, were included in each real-time RT-PCR assay. In addition, a standard titer of influenza A reference virus was included as a positive control in each assay.

The use of cell culture to isolate influenza virus from clinical specimens can result in substantial differences in recovery rates, depending on the protocol and cell line used. To compare the use of MDCK and Mv1Lu cells for swine influenza A virus isolation, we inoculated duplicate wells of each cell line separately with the nasal swab specimens. Interestingly, out of 16 influenza viruses isolated from either the abattoir or the diagnostic samples, 3 viruses could be recovered only from MDCK cells and another 3 isolates grew only on Mv1Lu cells. These viruses were subsequently iden-
tified as rH1N2 viruses or rH3N2 and rH1N2, respectively. It is not clear why these virus isolates had preferential growth in 1 of the 2 cell lines. One possible explanation for these findings could be subtle differences in receptor preferences of the virus isolates. To be able to detect a wide variety of influenza viruses at an equal level of sensitivity as in each individual cell line and to eliminate the need for separate cell lines, we mixed MDCK and MvILu cells at a ratio of 1:1. We found that each of the viruses that could previously be isolated in only 1 of the 2 cell lines was able to grow in the cell mixture, thus suggesting that the mixed cultures might serve as a useful alternative system for the isolation of influenza A viruses.

In the study reported here, we developed a rapid, sensitive, and highly specific real-time RT-PCR assay for the detection of influenza A viruses in pooled nasal swab specimens from pigs. In contrast to the time-consuming and labor-intensive procedures required for virus isolation in cell culture, results of the real-time RT-PCR assay can be obtained within a few hours. The real-time RT-PCR assay may replace virus isolation as a routine diagnostic test for swine influenza A viruses. However, virus isolation after screening with the real-time RT-PCR assay permits for further virus characterization, antigenic testing, and vaccine development.

References


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